

Waste water treatment plants as redistributors of resistance genes in bacteria

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Abstract

The aim of this study was to assess the number of both extended-spectrum β -lactamases (ESBL) producing *Enterobacteriaceae* and vancomycin-resistant enterococci (VRE) present in the inflow (hospital and urban sewage) and effluent waste water treatment plant (WWPT) and receiving waters.

The results show that the average counts of ESBL producing *Enterobacteriaceae* in hospital sewage were much higher (more than 2.5 logs) than those observed in urban waste water. On the other hand, the sewage treatment plant studied did not perform well in removing pathogenic microorganisms. Although *Enterobacteriaceae* concentrations were reduced in the final effluent by almost two log units as compared with the crude sewage concentrations, a high number of *Enterobacteriaceae* were detected in the effluent, representing a risk for microbiological pollution of water resources. A strong increase in ESBL-producing bacteria in the receiving water was detected after effluent discharge WWPT.

The results obtained have also demonstrated that the hospital sewage releases a high concentration of VRE ($> 3.0 \times 10^5$ CFU/100 ml), which represents 25% of the total population of enterococci presents in these waters. The hospital sewage contains a proportion of VRE 10 times higher in comparison with that detected in the urban sewage. The VRE proportion in the urban sewage was very low (2.6%) and constant along the different samplings. In the other hand, sewage discharged from municipal sewage treatment plant effluent also increased the VRE proportion in the river Sar water (1.5% of EVR in upstream to 3.8% downstream from the plant). In conclusion, sewage (particularly hospital sewage) contains a high proportion of ESBL producing *Enterobacteriaceae* and vancomycin resistant enterococci, implying a potential risk of spreading these



resistant bacteria and resistance genes to the environment and possibly to the human and animal population.

Keywords: *β -lactamases, drug resistance, ESBLs, hospital and urban sewage, Enterobacteriaceae, vancomycin-resistant enterococci.*

1 Introduction

During the last fifty years the utilization of antimicrobial drugs has steadily increased. Antimicrobials are extensively used in human medicine to treat or prevent bacterial infectious diseases. Antimicrobial resistant bacteria and antimicrobial residues are excreted from patients into the hospital wastewater daily [1]. Consequently, the hospital wastewater may be a hot spot for the propagation and dissemination of antimicrobial resistant and pathogenic bacterial isolates. Some reports showed that high resistance rates to several antimicrobial agents are also observed in commensal and environmental bacteria [2, 3]. Thus, the emergence and dissemination of antibiotic resistant bacteria in hospitals and environments is an emerging challenge to humankind.

Strains of *Enterobacteriaceae* that produce extended-spectrum β -lactamases have emerged as significant pathogens. First reported in the mid-1980s, they were mainly found in *Klebsiella pneumoniae* and *Escherichia coli*, although they can now be found in many other species [4]. The emergence of multiresistance in the *Enterobacteriaceae* family is a question that needs attention, because these are important causative agents of hospital infections, typically associated with pneumonias, blood stream infections, urinary tract infections, bacteremia and other intra-abdominal infections [5-8]. Reports of infection or colonization with ESBL-producing *Enterobacteriaceae* strains have focused mainly on hospitalized patients or nursing home residents [4]. However, ESBL-producing *Enterobacteriaceae* have also been described in animals and in patients in the community, with and without chronic conditions [9, 10]. Additionally, studies have demonstrated the ESBL-producing bacteria are isolated with increasing frequency in many parts of the world [7, 11-17].

Although many resistant bacteria have been isolated from environmental samples, there are only limited studies that have addressed the detection of ESBL-producing bacteria from these types of samples [18, 19]. Prado *et al* [19] observed the presence of *Klebsiella pneumoniae* producing ESBL in the effluent and sludge of hospital sewage plants. Therefore, the hospital sewage or the low efficacy of hospital sewage-treatment plants could result in the dissemination of ESBL-producing bacteria. In addition, it is generally accepted that waste water treatment plants play an important role in redistributing and disseminating antimicrobial resistance properties to bacteria. In fact, the almost permanent presence of detectable antimicrobial levels in the waters of waste water treatment plants could strongly influence the environment, creating a selective pressure that would be ultimately responsible for the dominance of populations of resistant microorganisms among those present in the habitat.

Although it was investigated the presence of ESBL-producing bacteria in hospital sewage and environmental samples, we lack data on the quantitative



importance of these bacteria in these environments, consequently the aim of this study was to quantify ESBL-producing *Enterobacteriaceae*, present in the inflow (hospital and urban sewage) and effluent waste water treatment plant (WWPT) and receiving waters.

By the other hand, enterococci have emerged as one of the clinical challenges when identified as the cause of serious or life threatening infections. Isolates of *Enterococcus faecalis* and *Enterococcus faecium* are the third- to fourth-most prevalent nosocomial pathogen worldwide [20]. Antimicrobial resistance in strains causing nosocomial infections is a growing problem and VRE in particular are considered a serious threat in hospitals around the world [21]. Vancomycin is often used as a last resort in treatment of antibiotic resistant gram-positive bacterial infections caused by organisms such as multi-resistant enterococci and methicillin resistant staphylococci. Infections due to VRE are associated with higher treatment costs, prolonged morbidity, and greater mortality rates [22].

In the USA, the prevalence of VRE is mainly documented as nosocomial infection in humans [23, 24]. The situation regarding VRE in Europe is diverse with prevalences ranging from <1 to >40% and many aspects of VRE acquisition and spread are still unknown [20]. In any case, acquired vancomycin resistance appears to be a serious and growing therapeutic challenge among enterococci all over Europe. According Werner *et al* [20], some EU countries have experienced an increasing VRE trend over time (e.g., Ireland, Germany, Greece); in other countries VRE prevalence is still low (e.g., in Nordic countries, the Netherlands); a few EU Member States showed decreasing VRE rates (e.g., Austria, Portugal, Italy).

The spread of these organisms from the hospital environment or other sources to environmental waters through discharged sewage or other means could increase the prevalence of these strains in the human population and become a potential risk to human health [25-27]. Several studies have detected VRE strains in hospital-impacted sewage [26, 28], sewage [29] and in receiving waters [28].

The second aim of the present study was to assess the number of vancomycin-resistant enterococci present in the hospital sewage and receiving waters.

2 Materials and methods

2.1 Water samples collection

Samples were collected from sewage effluent University Hospital of Santiago de Compostela (Spain), both wastewater and municipal sewage treatment plant of Santiago de Compostela, River Sar (upstream and downstream of sewage treatment plant discharge, sampling sites 1, 2 and 3, respectively) and estuary of Arousa (sampling site 4, shellfish cultivation area). Site 1, is at 0,2 Km upstream of WWPT, site 2 is at 7 Km downstream of WWPT and site 3 is at 15 Km downstream of WWPT.

Sampling was carried out in April 2004, September 2004, February 2005 and June 2005, over 2 weeks in each month. Wastewater and water samples were



collected into sterile bottles. The wastewater samples were collected during a 24h period, harvesting 50 ml every 60 min, refrigerated and analysed within 24h.

2.2 Isolation and enumeration of *Enterobacteriaceae*

The *Enterobacteriaceae* count from waste water samples were obtained by inoculating one ml aliquots of the appropriate dilution in chromogenic Coli-ID agar (supplied by bioMerieux, France). All plates were incubated aerobically for 24 ± 2 h at $36.0 \pm 1.0^\circ\text{C}$. All pink/violet and blue/grey colonies on Coli-ID agar were counted as presumptive *Enterobacteriaceae*. Typical colonies were picked off for confirmation as *Enterobacteriaceae* based on the following tests: Gram stain, oxidase and glucose fermentation [30].

The *Enterobacteriaceae* count from River Sar and estuary of Arousa were analysed by standard membrane filtration technique, using the same culture media and incubation conditions.

The same methods were employed for ESBL-producing *Enterobacteriaceae* counts, supplementing Coli-ID agar with 2 µg/ml of ceftazidime, cefotaxime, ceftriaxone and aztreonam. Confirmation of β -lactamase production by strains isolated from agar supplemented Coli-ID was carried out using the test specified by Steward *et al* [31].

2.3 Isolation and enumeration of enterococci

At the laboratory, wastewater samples (0.1 ml aliquots of the appropriate dilution) were directly streaked onto Slanetz & Bartley Medium for enterococci count. Water samples from River Sar and estuary of Arousa were analysed by membrane filter technique [32]. VRE were isolated on M-enterococcus containing 32 µg /ml vancomycin and were incubated for 48 ± 3 h at $36.0 \pm 1.0^\circ\text{C}$.

3 Results and discussion

The average counts of *Enterobacteriaceae* and ESBL-producing *Enterobacteriaceae* obtained from all wastewater samples (hospital and urban sewage, influent and effluent WWPT) collected throughout the study are shown in Figure 1. The results show that among the urban and hospital sewage there is little difference in the total level of enterobacteria. On the contrary, it is noteworthy that the average counts of ESBL producing *Enterobacteriaceae* in hospital sewage were much higher (more than 2.5 logs) than those observed in urban wastewater. This clearly highlights the importance of hospital sewage as a major source of antibiotic-resistant bacteria.

One of the recurring concerns because of the presence of ESBL-producing bacteria in these environments is associated with the transfer of conjugative plasmids, which also carry genes of resistance to aminoglycosides and sulfonamides, giving the bacteria multiresistance patterns [33]. Conjugative plasmids can be easily transferred to other bacteria [34, 35]. This mechanism of

gene transfer is particularly important in hospital effluents, because in such environments, the possibility of gene exchange is increased as a result of higher density and greater contact between these microorganisms, which allows for an increased rate of horizontal transfer of these genes.

The incorrect handling and disposal of the hospital sewage into inappropriate locations may compromise public health by contaminating the soil, water supplies or recreational waters, and facilitate dissemination of microorganisms and resistance genes into the environment. In this way, a high prevalence of resistant micro-organisms in hospitals shows a potential risk for disseminating pathogenic agents into the environment, particularly through the wastewaters.

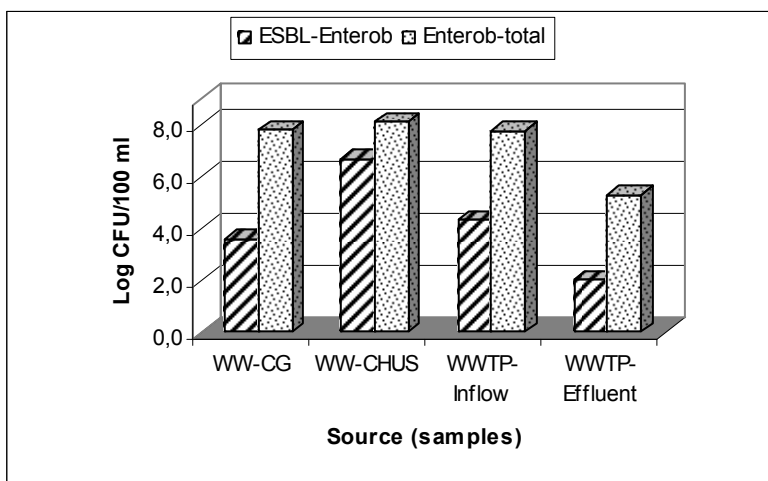


Figure 1: Average counts of *Enterobacteriaceae* and ESBL producing *Enterobacteriaceae* obtained from the water samples. WW-CG, waste water general collector of Santiago de Compostela. WW-CHUS, waste water from University hospital complex of Santiago de Compostela. WWTP, waste water treatment plant.

By the other hand, the sewage treatment plant studied did not perform well in removing pathogenic micro-organisms. Although *Enterobacteriaceae* concentrations were reduced in the final effluent by almost two log units as compared with the crude sewage concentrations, a high number of *Enterobacteriaceae* were detected in the effluent, representing a risk for microbiological pollution of water resources. Thus, as shown in Figure 2, the wastewater discharge from WWTP causes a strong increase in ESBL-producing bacteria in the receiving waters, so that 15 km downstream of discharge point, is still detected a high level of resistant bacteria. In Figure 2, also notes the presence of ESBL-producing bacteria in the water of shellfish cultivation area affected by the river waters at that WWTP discharged wastewater. Thus, the prevalence of ESBL-producing *Enterobacteriaceae* was notable in all the environments studied, suggesting a global expansion of these enzymes, as recorded Mesa *et al* [18].

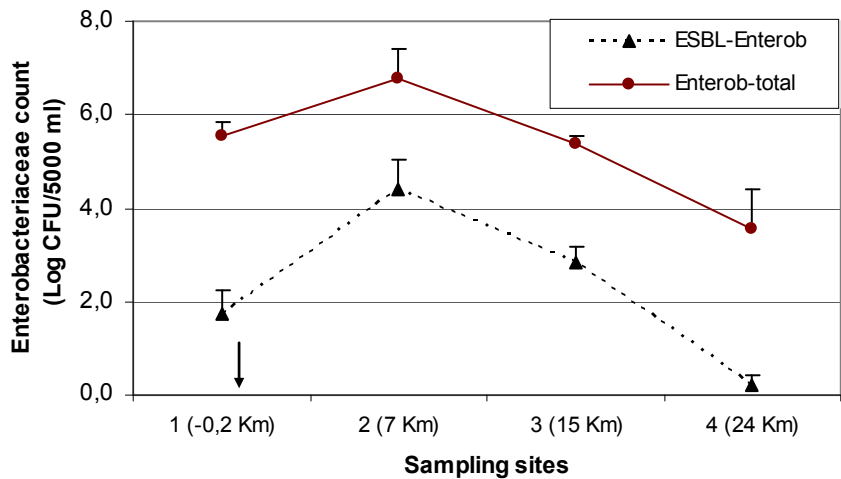


Figure 2: *Enterobacteriaceae* (Enterob-total) and ESBL producing *Enterobacteriaceae* (ESBL-Enterob) counts in four sampling sites along the river Sar. Values shown are geometric means of six determinations. The arrow indicates the dumping site of treatment plant into the river.

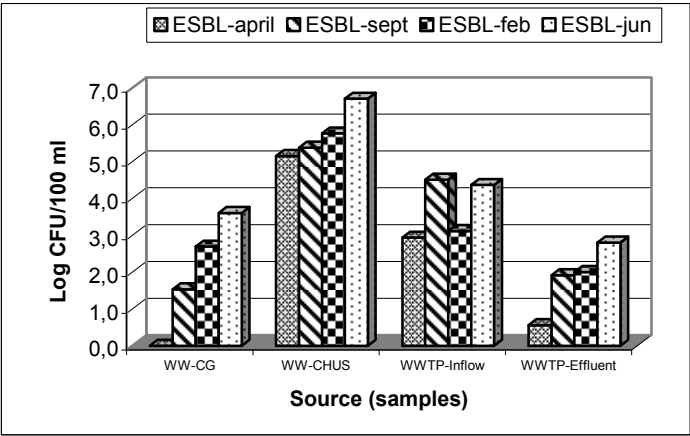


Figure 3: ESBL-producing *Enterobacteriaceae* (ESBL) counts obtained in water samples collected in April, September (sept), February (feb) and June (jun). WW-CG, waste water general collector of Santiago de Compostela. WW-CHUS, waste water from University hospital complex of Santiago de Compostela. WWTP, waste water treatment plant.



When analyzing the results is done taking into account the time of year, Figure 3 shows that the release of ESBL-producing *Enterobacteriaceae* from hospital waste water was very high throughout the year, particularly in the period of time for June. This same behaviour was observed in waste after samples collected in the other three points. A temporal distribution of ESBL-producing *Enterobacteriaceae* was also observed by Mesa *et al* [18] in Spain, who found a higher prevalence between July and November and lowest between February and May.

The results obtained have demonstrated that the hospital sewage also release a high concentration of VRE ($> 3.0 \times 10^5$ CFU/100 ml), which represents 25% of the total population of enterococci presents in these waters (Figure 4). The hospital sewage contains a proportion 10 times higher of VRE in comparison with the one that was detected in the urban sewage. In Figure 4 also may be observed that the proportion of VRE was very similar in influent and effluent of the waste water treatment plant.

The VRE proportion compared to the total population of Enterococci in the hospital wastewater throughout the year ranged between 37.05% in April and 17.6% in February. Nevertheless, the median values were constant along year (Figure 5). The VRE proportion in the urban sewage was very low (2.6%) and constant along the different samplings. In the other hand, sewage discharged from municipal sewage treatment plant effluent increased the VRE proportion in the river Sar water (1.5% of EVR in upstream to 3.8% downstream from the plant). The results obtained shown that the presence of VRE in environment aquatic is clearly influenced by hospital sewage, which are led to municipal treatment plants and mixed with urban sewage (Figure 6).

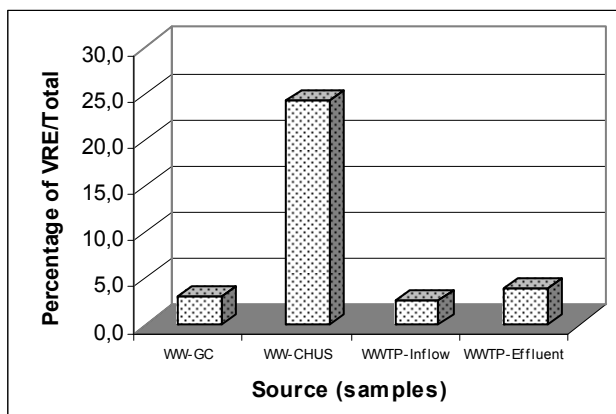


Figure 4: Proportion of vancomycin-resistant Enterococci (VRE) compared to the total population of Enterococci obtained in the water samples. WW-CG, waste water general collector of Santiago de Compostela. WW-CHUS, waste water from University hospital complex of Santiago de Compostela. WWTP, waste water treatment plant.

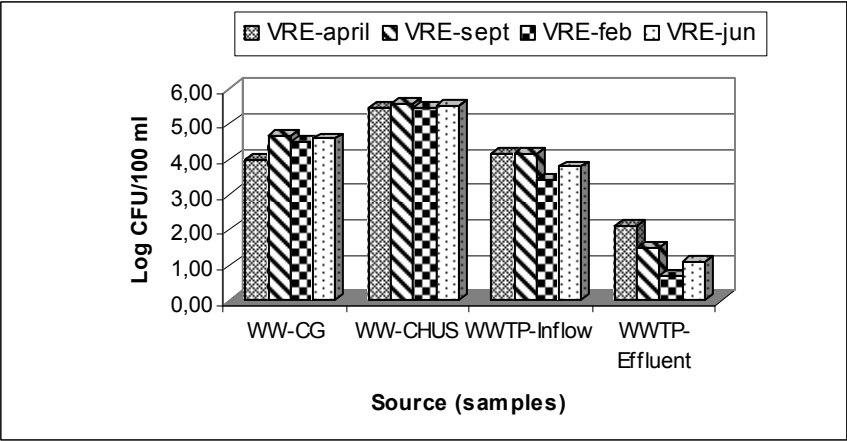


Figure 5: Vancomycin-resistant Enterococci (VRE) counts obtained in water samples collected in April, September (sept), February (feb) and June (jun). WW-CG, waste water general collector of Santiago de Compostela. WW-CHUS, waste water from University Hospital Complex of Santiago de Compostela. WWTP, waste water treatment plant.

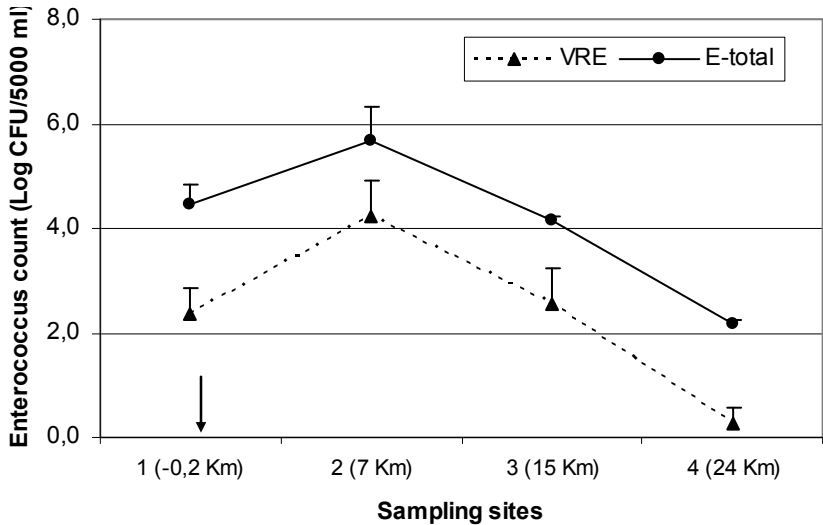


Figure 6: Vancomycin-resistant enterococci (VRE) and total enterococci (E-total) counts in four sampling sites along the river Sar. Values shown are geometric means of six determinations. The arrow indicates the dumping site of treatment plant into the river.



In conclusion, sewage (particularly, sewage hospital) contains a high proportion of ESBL-producing *Enterobacteriaceae* and vancomycin resistant enterococci, implying a potential risk of spreading these resistant bacteria and resistance genes to the environment and possibly to the human and animal population. The high incidence of resistant bacteria in sewage implies a need for high efficient hygienic treatment of sewage (application of final disinfection processes for to reduce the microbial burden), with the intention of minimizing the impact from the release of hospital wastewaters into water resources, preventing dissemination of multiresistant microorganisms and their genes of resistance into the environment, thus promoting prevention measures to protect public health.

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